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# Mutation-dependent activity of BI4020, JND3229, and TQB3804 in an EGFR mutant Ba/F3 cell panel

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## Abstract

Background: EGFR C797S impairs the activity of covalent EGFR inhibitors. Several fourth-generation inhibitors have been developed, but comparisons across different experimental systems do not adequately define their relative activity against individual EGFR genotypes.

Methods: Osimertinib, BI4020, JND3229, and TQB3804 were evaluated in Ba/F3 cells expressing wild-type EGFR or nine mutant variants. Cell viability was assessed by an MTT assay after 96 h of exposure. EGFR and AKT phosphorylation was examined in L858R/C797S-expressing cells after treatment with the inhibitors.

Results: Osimertinib $IC_{50}$ values were 1.39–2.84 nM in cells expressing activating mutations with or without T790M, but 419.42–1422.52 nM in cells expressing activating mutations together with C797S. In 19Del/T790M/C797S and L858R/T790M/C797S cells, BI4020 $IC_{50}$ values were 0.39 and 0.20 nM, respectively, compared with 61.18 and 13.59 nM for JND3229 and 3.85 and 3.42 nM for TQB3804. In L858R/C797S cells, BI4020 and JND3229 showed similar mean $IC_{50}$ values of 1.40 and 1.49 nM. TQB3804 showed lower $IC_{50}$ values in C797S compound mutants containing T790M than in the corresponding models without T790M. BI4020, JND3229, and TQB3804 reduced EGFR and AKT phosphorylation in L858R/C797S cells, whereas osimertinib showed little apparent suppression under the same conditions.

Conclusions: The three fourth-generation inhibitors exhibited distinct genotype-associated activity profiles. These findings support evaluating the complete EGFR mutation profile when comparing C797S-active inhibitors, while further validation in human lung cancer models is required.

## Introduction

EGFR mutations define a therapeutically important subset of non-small-cell lung cancer (NSCLC). Osimertinib demonstrated improved progression-free survival over first-generation EGFR tyrosine kinase inhibitors (TKIs) in untreated EGFR-mutant advanced NSCLC [1]. Nevertheless, acquired resistance remains a barrier to sustained disease control. Among EGFR-dependent resistance mechanisms, substitution of cysteine 797 by serine is particularly relevant because this residue participates in covalent inhibitor binding. C797S can therefore compromise the activity of osimertinib despite persistence of an activating EGFR mutation [2].

The biological and therapeutic meaning of C797S depends on its accompanying mutations. An activating mutation combined with C797S but without T790M differs from an activating mutation combined with both T790M and C797S. When T790M is present, its allelic relationship to C797S also affects the activity of subsequent treatment strategies [3]. Thus, C797S positivity alone does not fully characterize a drug target. Both exon 19 deletion (19Del) and exon 21 L858R backgrounds, and the presence or absence of T790M, should be considered when evaluating candidate inhibitors.

BI4020, JND3229, and TQB3804 have been reported as C797S-active fourth-generation EGFR inhibitors [4–7]. BI4020 is a noncovalent macrocyclic inhibitor developed to preserve mutant EGFR activity while reducing wild-type EGFR inhibition [4]. JND3229 is a reversible inhibitor with activity against C797S-containing EGFR mutants [5]. TQB3804 demonstrated activity against triple-mutant EGFR in preclinical studies [6,7]. These development reports establish the rationale for testing the compounds, but values generated using different biochemical assays, cell systems, and exposure conditions cannot be directly interpreted as a comparative ranking.

Our group previously demonstrated molecular differences between 19Del- and L858R-expressing Ba/F3 cells using proteomic, transcriptomic, and metabolomic analyses [8]. We also used EGFR-mutant Ba/F3 models to investigate glutamine-related vulnerabilities in osimertinib-resistant cells [9]. These studies provide a basis for examining how EGFR mutation context is associated with drug response in a shared cellular system. Here, we compared osimertinib with BI4020, JND3229, and TQB3804 across a panel of wild-type and mutant EGFR-expressing Ba/F3 cells and assessed EGFR–AKT signaling in the L858R/C797S model.

## Materials and methods

### Reagents

Osimertinib (Selleck Chemicals, Houston, TX, USA), BI4020 (Catalog No. S8921, Selleck Biotechnology, Kanagawa, Japan), JND3229 (Catalog No. S6881, Selleck Biotechnology), and TQB3804 (Catalog No. S6698, Selleck Biotechnology) were dissolved in dimethyl sulfoxide (DMSO) and were used for the drug-sensitivity and signaling experiments.

### Cell models and culture

Ba/F3 is a murine interleukin (IL)-3-dependent pro-B cell line (RIKEN Cell Bank, Tsukuba, Japan). Ba/F3 cells were cultured in RPMI-1640, with 10% FBS, 5 ng/mL recombinant murine IL-3 (Cell Signaling Technology), and 1% penicillin/streptomycin in a tissue culture incubator at 37°C under 5% $CO_2$. EGFR expression vectors were constructed by inserting the full-length RT–PCR product of *EGFR* into the pMX-EF1-Bsd vector (Invitrogen, Carlsbad, CA, USA), together with enhanced GFP, to monitor expression of the inserts. The mutation was generated by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, CA, USA) and confirmed by sequencing. Ten EGFR (wild type, 19Del, 19Del/T790M, L858R, L858R/T790M, 19Del/T790M/C797S, L858R/T790M/C797S, 19Del/C797S, L858R/C797S, C797S) expression vectors with pCMV-gp and pCMV-VSVG were introduced into Ba/F3 cells using Lipofectamine 3000 (ThermoFisher Scientific, Waltham, MA, USA). After 48 h of transfection, cells were cultured with blasticidin (10 μg/mL), with IL-3 for wild type and C797S without IL-3 for others. Stable EGFR-expressing clones were isolated by limiting dilution. Confirmation of Ba/F3 cells with *EGFR* mutations was performed by RT–PCR and sequencing. The transfected Ba/F3 cell lines were EGFR wild type (WT), 19Del (M1), 19Del/T790M (M2), L858R (M3), L858R/T790M (M4), 19Del/T790M/C797S (M5), L858R/T790M/C797S (M6), 19Del/C797S (M7), L858R/C797S (M8), and C797S (M9). Some of the variants were used in our previous studies [8,9]. The present report focuses on drug sensitivity and signaling responses rather than a new characterization of the cell panel.

**Cell viability assay**

Drug sensitivity was evaluated using a modified MTT assay [10]. Cells were seeded in 96-well plates at 1,500 cells per well and continuously exposed to each inhibitor for 96 h. Each experiment was performed in triplicate or quadruplicate, and three independent experiments were conducted on different days. The concentration producing 50% inhibition of the viability-related MTT signal ($IC_{50}$) based on three independent experiments was used to evaluate each drug sensitivity, and values are reported in nM as mean ± standard deviation (SD).

**Immunoblotting**

M8 cells expressing EGFR L858R/C797S were exposed to osimertinib, BI4020, JND3229, or TQB3804 at 100 nM for 4 h. Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, #78501) containing Halt Phosphatase Inhibitor Cocktail (Thermo Scientific, #1861277) and Protease Inhibitor Cocktail Kit (Thermo Scientific, #78410). Proteins were separated on polyacrylamide gels prepared using the TGX FastCast Acrylamide Kit (Bio-Rad, #1610173) and transferred to Immobilon-P polyvinylidene difluoride membranes (Merck Millipore, IPVH00010). Membranes were probed for phosphorylated (p)EGFR, total EGFR, pAKT, total AKT, and GAPDH. Antibodies to EGFR (Cell Signaling #2232; 1:2,000 dilution), pEGFR (Cell Signaling #3777, 1:3,000 dilution), pAKT (Cell Signaling #13038, 1:2,000 dilution), AKT (Cell Signaling #9272, 1:4,000 dilution), and GAPDH (14C10) rabbit monoclonal antibody (Cell Signaling ＃2118; 1:10,000 dilution) were purchased. Signals were detected using SuperSignal West Pico PLUS chemiluminescence reagent (Thermo Scientific, #34577).

**Declaration of generative AI and AI assisted technologies**

During preparation of this manuscript, the authors used ChatGPT (OpenAI) to assist with organization of the English text and identification of relevant literature. No primary experimental data were generated using this tool. The authors will review and verify the scientific interpretations, numerical values, and references before submission and take responsibility for the final content.

## Results

### C797S was associated with reduced osimertinib sensitivity

The sensitivity profiles of the EGFR-expressing cell panel are presented in Table 1. Cells expressing M1, M2, M3, or M4 had low osimertinib $IC_{50}$ values of 2.84, 1.41, 2.80, and 1.39 nM, respectively. By contrast, $IC_{50}$ values were 663.38 and 1422.52 nM in the M5 and M6 cells, and 419.42 and 670.62 nM in the corresponding C797S cells lacking T790M (M7 and M8, respectively). This profile is consistent with reduced osimertinib activity in the presence of C797S. As an example, Figure 1 shows the dose-response curves for the drugs in M8 cells.

### Fourth generation inhibitors displayed distinct mutation associated profiles

BI4020 had the lowest mean $IC_{50}$ among the four inhibitors in both triple-mutant models: 0.39 ± 0.24 nM in M5 and 0.20 ± 0.05 nM in M6. TQB3804 also showed low $IC_{50}$ values in these models (3.85 ± 0.61 and 3.42 ± 0.21 nM, respectively). JND3229 $IC_{50}$ values were 61.18 ± 3.08 nM in M5 and 13.59 ± 1.35 nM in M6. Each fourth-generation inhibitor had a lower mean $IC_{50}$ than osimertinib in these models.

In the absence of T790M, BI4020 $IC_{50}$ values were 9.10 ± 2.11 nM in 19Del/C797S cells and 1.40 ± 0.31 nM in L858R/C797S cells. JND3229 values were 34.82 ± 4.36 and 1.49 ± 0.17 nM, respectively. Accordingly, BI4020 and JND3229 had similar mean activity in M8, whereas BI4020 had a lower mean $IC_{50}$ in M7. TQB3804 values were 71.06 ± 0.41 nM in M7 and 15.01 ± 4.07 nM in M8, approximately 18.5- and 4.4-fold higher than in the corresponding triple-mutant models. Conversely, the JND3229 $IC_{50}$ in L858R/T790M/C797S cells was approximately 9.1-fold higher than that in L858R/C797S cells. These observations indicate that the presence of T790M was associated with different activity patterns for different compounds.

The activating-mutation background also influenced the observed profiles. JND3229 had a lower mean $IC_{50}$ in L858R/C797S than in 19Del/C797S cells, and in L858R/T790M/C797S than in 19Del/T790M/C797S cells. BI4020 values were lower in the L858R/C797S model than in the 19Del/C797S model, whereas TQB3804

showed similar mean values in the two triple-mutant backgrounds. These comparisons are descriptive and were not tested for statistical significance.

**Responses in wild type EGFR and C797S only models**

In the wild-type EGFR-expressing cells, $IC_{50}$ values were 1567.87 ± 176.68 nM for osimertinib, 369.30 ± 13.19 nM for BI4020, 140.93 ± 26.22 nM for JND3229, and 493.10 ± 19.78 nM for TQB3804. C797S-only cells had values of 1519.85 ± 70.25, 604.30 ± 17.43, 70.20 ± 2.50, and 917.20 ± 55.30 nM, respectively. These control-model responses are reported separately because the growth-factor requirements and EGFR dependence of these models must be established before their values can be interpreted as measures of mutant selectivity.

**Suppression of EGFR and AKT phosphorylation in L858R C797S cells**

Figure 2 shows the immunoblot results for M8 cells treated at 100 nM for 4 h. BI4020, JND3229, and TQB3804 markedly reduced the visible pEGFR and pAKT signals compared with the control. Osimertinib showed little apparent reduction at the same concentration. Total EGFR, total AKT, and GAPDH signals remained detectable across the treatment lanes. The signaling findings are consistent with the viability-assay results in M8, although a single concentration and time point do not establish comparative signaling potency or the duration of inhibition.

## Discussion

This study provides a comparison of three C797S-active inhibitors under a shared viability-assay framework. The main finding is that BI4020, JND3229, and TQB3804 showed distinct activity profiles across the EGFR mutant panel. BI4020 had the lowest mean $IC_{50}$ in both triple-mutant models and remained active in C797S compound mutants lacking T790M. JND3229 approached BI4020 activity in L858R/C797S cells, while TQB3804 was more active in the C797S models containing T790M than in the corresponding models lacking T790M. The value of this dataset lies in resolving these genotype-associated differences within one experimental platform.

The comparison between double- and triple-mutant models is particularly informative. T790M was associated with lower TQB3804 $IC_{50}$ values in both activating-mutation backgrounds, but with a higher

JND3229 $IC_{50}$ in the L858R/C797S background. These observations should not be interpreted as evidence that a resistance mutation universally sensitizes or desensitizes EGFR to fourth-generation inhibitors. Instead, they suggest that the contribution of an additional kinase-domain substitution is compound dependent. Differences in binding interactions, intracellular exposure, or model-specific EGFR expression could contribute, and the present experiments do not distinguish among these explanations. Verification using independent clones and biochemical measurements would help determine which differences arise directly from the mutant kinase.

The broad activity of BI4020 is consistent with its development as a noncovalent macrocyclic EGFR inhibitor [4]. Structural work has further examined its interactions with the kinase domain and the basis of mutant-associated activity [11]. A subsequent study showed activity against selected more complex resistance mutants, while also demonstrating that activity is not uniform across all EGFR variants [12]. Our results extend the comparative context to both 19Del and L858R backgrounds with or without T790M and C797S. However, the low $IC_{50}$ values in our Ba/F3 models cannot establish clinical efficacy, achievable target coverage, or superiority over other candidates in patients.

JND3229 illustrates why C797S-active inhibitors should be evaluated beyond the triple-mutant setting. Its low mean $IC_{50}$ in L858R/C797S cells contrasts with the higher values in the two triple-mutant models. The original report described cellular $IC_{50}$ values of 0.51 μM for L858R/T790M/C797S and 0.32 μM for 19Del/T790M/C797S Ba/F3 cells [5]. These absolute values and their relative ordering differ from our results. The cause cannot be established from summary data; differences in cell derivation, expression levels, culture conditions, assay readout, or exposure duration are possible contributors. The discrepancy supports independent replication and caution when comparing values across laboratories, rather than a claim that our assay demonstrates greater intrinsic potency.

TQB3804 showed a different pattern. Its similar mean $IC_{50}$ values in the two triple-mutant models are consistent with the activity against both triple-mutant kinases reported in its preclinical evaluation [6]. Its higher values in C797S compound mutants without T790M suggest that activity in the triple-mutant setting should not automatically be extrapolated to every C797S-containing genotype. The primary TQB3804 evidence cited here is a conference abstract, and its quantitative findings should be distinguished from a complete peer-reviewed

development report. Our cellular findings complement that evidence but do not provide structural proof of a T790M-dependent interaction.

The activating EGFR mutation is another relevant component of this comparison. In our previous multi-omics study, 19Del- and L858R-expressing Ba/F3 cells displayed different molecular and metabolic features [8]. The present drug-response differences are compatible with the broader idea that these backgrounds are biologically distinct, but do not establish that metabolic differences account for inhibitor sensitivity. Our subsequent work on SLC1A3 and glutaminase showed that osimertinib-resistant models can have additional metabolic vulnerabilities [9]. Together, the studies support considering mutation-specific drug inhibition and cellular adaptation as complementary research questions. No metabolic measurements or combination-treatment experiments were performed in the present dataset.

Recent systematic variant profiling further supports the importance of the activating EGFR background. Lee et al. used prime editing to characterize kinase-domain variants in an L858R-based NSCLC model and integrated these results with a published exon 19 deletion-based dataset, identifying both shared and background-specific resistance patterns to afatinib, dacomitinib, and osimertinib [13]. This finding provides a broader context for our observation that inhibitor responses differed between 19Del- and L858R-containing C797S models. Their study did not evaluate BI4020, JND3229, or TQB3804, and therefore does not directly validate the activity profiles reported here. Nevertheless, the two approaches are complementary: systematic variant screening maps the wider resistance landscape, whereas our focused comparison examines selected C797S-containing genotypes under a shared assay framework. Extending such profiling to C797S-active inhibitors could help define how additional kinase-domain substitutions modify their activity.

The M8 (L858R/C797S) immunoblot provides qualitative evidence of pathway suppression accompanying viability inhibition. The reduction of pEGFR and pAKT by all three fourth-generation compounds is consistent with inhibition of EGFR-associated signaling in this model. However, 100 nM exceeds the M8 viability $IC_{50}$ of each compound, and the experiment therefore cannot differentiate their signaling potencies. The 4-h signaling endpoint also differs from the 96-h MTT endpoint. Dose-dependent

immunoblotting and repeated measurements would be needed to relate target inhibition more closely to the viability response. These results do not demonstrate apoptosis or irreversible cell killing.

Several limitations define the scope of the findings. Ba/F3 cells are engineered murine hematopoietic cells and do not reproduce the lineage context, co-mutations, or heterogeneous resistance pathways of human lung cancer. The activity profiles may be affected by expression levels and clonal differences. Wild-type EGFR and C797S-only controls require particular caution because their growth-factor dependence may differ from that of activating-mutant models. Consequently, ratios using these control $IC_{50}$ values should not be described as clinical therapeutic windows without validating comparable EGFR-dependent growth. No human lung cancer validation, animal efficacy, pharmacokinetic analysis, or formal statistical comparison is included.

In conclusion, BI4020, JND3229, and TQB3804 inhibited C797S-containing activating EGFR mutants but showed different genotype-associated activity profiles. BI4020 had the lowest mean $IC_{50}$ in the two triple-mutant models, JND3229 showed particularly low values in L858R/C797S cells, and TQB3804 showed greater activity in triple mutants than in the corresponding C797S models lacking T790M. These results provide a focused comparative basis for further validation and support interpreting C797S-active inhibitor responses in the context of the complete EGFR genotype.

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## Competing Interests

Dr. Takigawa has received grants and personal fees from Eli Lilly Japan, Chugai Pharmaceutical, and Boehringer Ingelheim Japan, and personal fees from MSD, Nippon Kayaku, Ono Pharmaceutical, and Bristol Myers Squibb Japan outside the submitted work. The other authors have no conflicts of interest to declare.

## Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ayaka Mimura, Masami Takeyama, Nobuaki Ochi, Yasunari Nagasaki, Tomoko Ikeda and Nagio Takigawa. The first draft of the manuscript was written by Nagio Takigawa and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

## Table 1

$IC_{50}$ values for osimertinib and fourth-generation EGFR inhibitors in the EGFR-expressing Ba/F3 cell panel

| EGFR variant | Osimertinib | BI4020 | JND3229 | TQB3804 |
|---|---|---|---|---|
| WT | 1567.87 ± 176.68 | 369.30 ± 13.19 | 140.93 ± 26.22 | 493.10 ± 19.78 |
| M1 19Del | 2.84 ± 0.15 | 8.51 ± 2.28 | 14.02 ± 2.95 | 30.55 ± 6.20 |
| M2 19Del/T790M | 1.41 ± 0.08 | 0.22 ± 0.02 | 81.33 ± 13.05 | 1.60 ± 0.45 |
| M3 L858R | 2.80 ± 0.02 | 0.99 ± 0.01 | 35.40 ± 0.55 | 6.01 ± 0.20 |
| M4 L858R/T790M | 1.39 ± 0.13 | 0.15 ± 0.03 | 16.48 ± 0.92 | 1.32 ± 0.05 |
| M5 19Del/T790M/C797S | 663.38 ± 37.28 | 0.39 ± 0.24 | 61.18 ± 3.08 | 3.85 ± 0.61 |
| M6 L858R/T790M/C797S | 1422.52 ± 74.47 | 0.20 ± 0.05 | 13.59 ± 1.35 | 3.42 ± 0.21 |
| M7 19Del/C797S | 419.42 ± 22.08 | 9.10 ± 2.11 | 34.82 ± 4.36 | 71.06 ± 0.41 |
| M8 L858R/C797S | 670.62 ± 26.85 | 1.40 ± 0.31 | 1.49 ± 0.17 | 15.01 ± 4.07 |
| M9 C797S | 1519.85 ± 70.25 | 604.30 ± 17.43 | 70.20 ± 2.50 | 917.20 ± 55.30 |

Values are mean ± SD in nM after 96 h of drug exposure of three independent experiments. IL-3 was included in the culture medium during the MTT assay for WT cells and M9 cells expressing EGFR C797S. WT, wild-type EGFR; 19Del, exon 19 deletion.

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Figures

Figure 1

Dose–response profiles of EGFR inhibitors in Ba/F3 cells expressing EGFR L858R/C797S.

M8 cells were treated with (A) osimertinib, (B) BI4020, (C) JND3229, or (D) TQB3804 at the indicated concentrations for 96 h. Cell viability was assessed using an MTT assay and normalized to the untreated control. Data are presented as the mean ± SEM of three independent experiments. Drug concentrations are expressed in nM on a logarithmic scale.

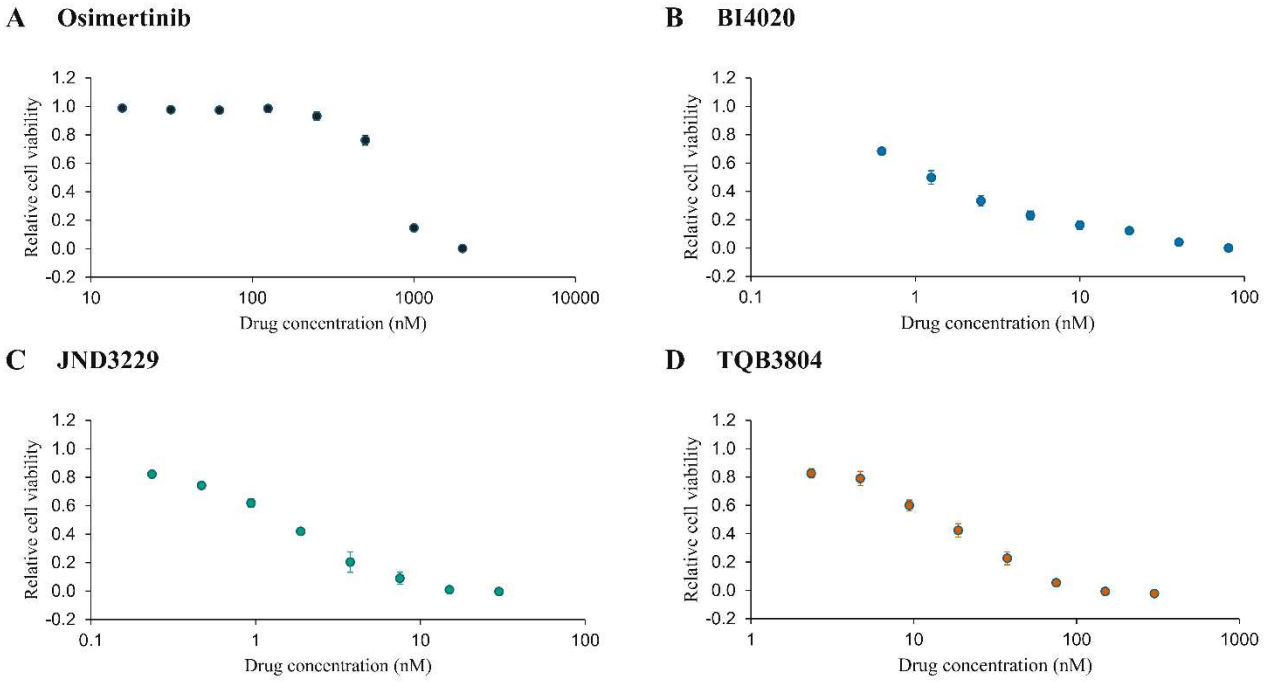


Figure 2. EGFR and AKT phosphorylation in EGFR L858R/C797S-expressing Ba/F3 cells following EGFR inhibitor exposure. M8 cells were treated with osimertinib, BI4020, JND3229, or TQB3804 at 100 nM for 4 h. Immunoblotting was performed in two independent experiments using protein lysates prepared on separate days. Similar results were obtained, and representative blots are shown.

